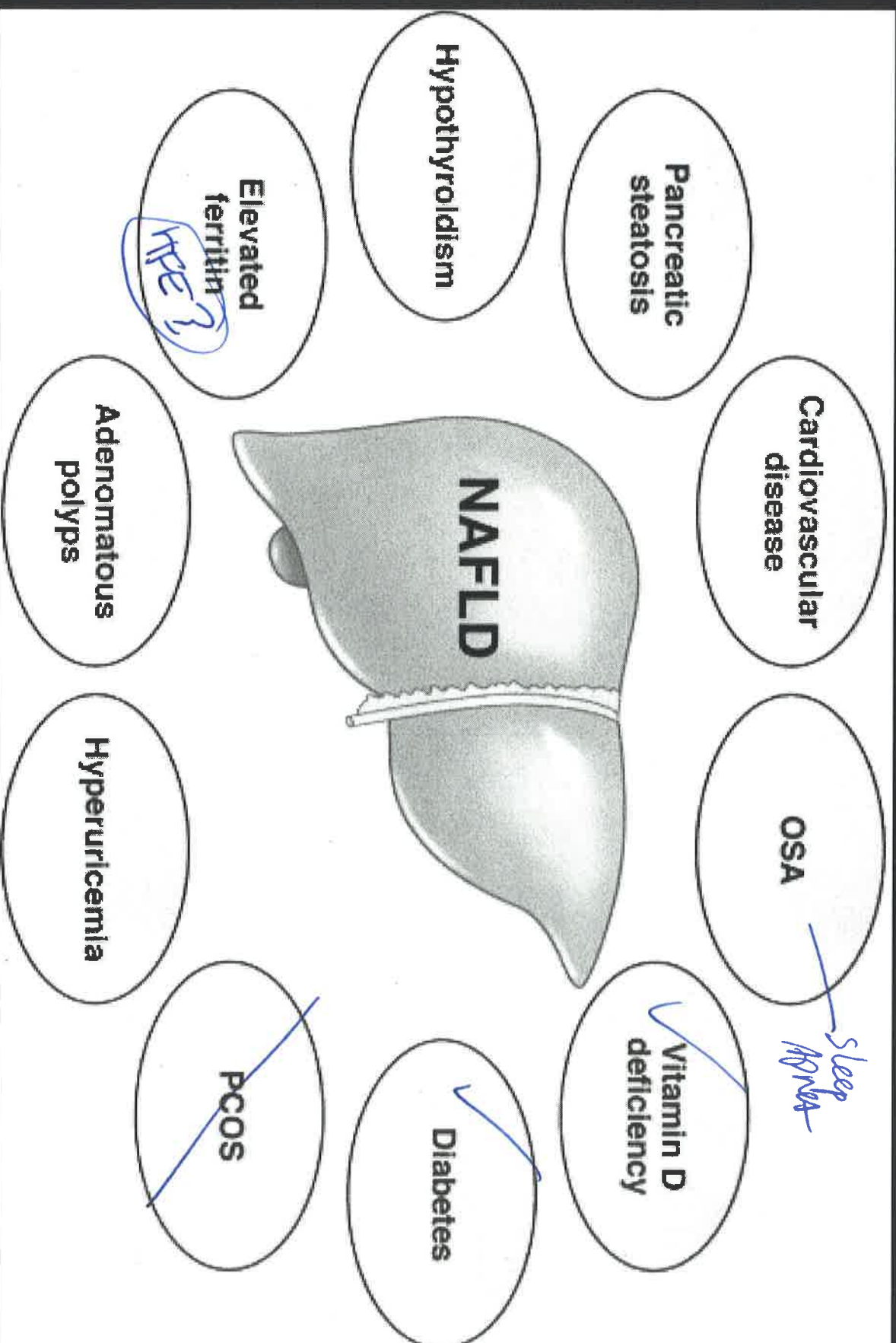


NAFLD: Clinical



Ongoing RCTs on NASH

Antifibrotic
Monoclonal
antibody against
lysyl oxidase like
molecule 2

Agent		Duration	Estimated enrolment (pts)	Population specifics	Clinicaltrials.gov identifier
Ethylcosapentate (EPA-E) [15]	1 mg vs. placebo, oral	52 weeks	243	Non-cirrhotics	NCT01154985
Obethicolic acid [120]	1 mg vs. placebo, oral	72 weeks	280	Non-cirrhotics	NCT01265498
Simtuzumab (GS 6624)	75 mg vs. 120 mg vs. placebo, intravenous	96 weeks	225	Cirrhosis	NCT01672866
Simtuzumab (GS 6624)	200 mg vs. 700 mg vs. placebo, subcutaneous	96 weeks	225	Advanced fibrosis without cirrhosis	NCT01672879
GFT 505	80 mg vs. 120 mg vs. placebo, oral	52 weeks	270	Non-cirrhotics	NCT01694849
Liraglutide	1.8 mg OD vs. placebo	48 weeks	50	Both diabetic and non-diabetic patients	NCT01237119
Losartan	50 mg vs. placebo	2 years	214	NASH with fibrosis	NCT01051219
Cenicriviroc	150 mg vs. placebo	2 years	252	NASH with fibrosis, no cirrhosis	NCT002217475
Aramchol	400 mg vs. 600 mg vs. placebo	1 year	240	NASH with prediabetes/diabetes and overweight/visceral adiposity	NCT002279524



Nonalcoholic fatty liver disease: new treatments

Timothy Hardy, Quentin M. Anstee, and Christopher P. Day

Purpose of review

Nonalcoholic fatty liver disease is the most common cause of liver dysfunction in the western world because of its close association with obesity, insulin resistance and dyslipidaemia. Nonalcoholic steatohepatitis (NASH) is a particular health concern due to the increased morbidity and mortality associated with progressive disease. At present, without specific targeted pharmacological therapies, the mainstay of therapy remains weight loss through dietary modification and lifestyle change; thus, the purpose of this review is to summarize the recent evidence for current and emerging therapies in NASH.

Recent findings

Some existing medications, including pioglitazones and angiotensin receptor antagonists, may be repurposed to help treat this condition. Vitamin E may improve histology in NASH, but safety issues limit its use. Recently, a number of novel agents specifically targeting nonalcoholic fatty liver disease pathogenesis have entered clinical trials, including the farnesoid X receptor agonist obeticholic acid, which has shown significant histological improvements in steatohepatitis and fibrosis.

Summary

Diet/lifestyle modification remains the mainstay of treatment. For patients with NASH and advanced fibrosis, current liver-directed pharmacotherapy with vitamin E and pioglitazone offer some benefits; obeticholic acid appears promising and is currently being tested. Comorbidities must be diagnosed and treated; cardiovascular disease remains a primary cause of death in these patients.

Keywords

nonalcoholic fatty liver disease, nonalcoholic steatohepatitis, treatment

INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is the most common cause of liver dysfunction in the western world [1] because of its close association with obesity, insulin resistance and dyslipidaemia; it is therefore considered the hepatic manifestation of the metabolic syndrome. A particular health concern is patients with nonalcoholic steatohepatitis (NASH) with accompanying hepatocellular injury that can lead to progressive liver fibrosis, cirrhosis and hepatocellular carcinoma (HCC) as well as increased cardiovascular risk [1]. At present, there is no approved therapy for NASH and the optimal treatment remains uncertain; effective therapies are thus a research priority to reduce the anticipated burden of liver disease.

THERAPY

The rationale for therapeutic approaches is centred on the concept that while simple steatosis has not been associated with morbidity, NASH is associated with a more than 10-fold increased risk of liver-related death (2.8 vs. 0.2%) and a doubling of

cardiovascular risk [2]; at the time of diagnosis, 25–33% of patients with NASH have advanced fibrosis, including cirrhosis [3,4]. After adjustment for confounders, NASH has a similar fibrotic potential to that of chronic hepatitis C [3,4]. Pooled data suggest that about 21% of patients with NASH will have some regression of fibrosis while 38% of patients will progress over 5.3 years' follow-up [3], results that have recently been confirmed in a dual-biopsy Northern European population [5^{***}].

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KEY POINTS

- Diet and lifestyle modification, with weight loss, remain the mainstay of treatment for NAFLD.
- Vitamin E and pioglitazone offer some benefit in selected cases (NASH and bridging fibrosis), although these must be balanced with potential adverse effects.
- Obeticholic acid may provide the first liver-targeted therapy for NASH.
- Cardiovascular disease must be diagnosed and treated.

Lifestyle modifications

Weight reduction is recommended as the initial step in management of NASH. Pharmacological agents such as orlistat may help achieve weight loss; however, whether these confer additional independent benefit beyond that due to weight loss is unclear [6,7]. Lifestyle modification therefore remains the primary therapy for weight reduction, particularly in the absence of approved pharmacotherapy; it encompasses diet, physical activity (aerobic and resistance) and behavioural change, or a combination of all three. Trial evidence shows that weight reduction more than 7% sustained over 48 weeks is associated with significant reduction in histological severity of NASH [8]. A systematic review of the available evidence for lifestyle modifications in NAFLD has recently been conducted [9]. Less than 50% of patients achieve the necessary weight loss goal of more than 7% in the trial setting [8], and many have questioned the sustainability of this type of intervention [10]. Resistance training, which is less burdensome on the cardiovascular system,

shares the metabolic improvements seen in more strenuous aerobic exercise and may be more sustainable [11]. Nevertheless, in the many patients that fail to implement lifestyle changes or have advanced disease (bridging fibrosis) on index biopsy, specific liver-directed pharmacotherapy may be needed. No drugs are currently licensed specifically for treating NASH; there is an urgent need for well designed randomized controlled trials (RCTs) with appropriate endpoints to narrow this gap. Table 1 [12–21, 22^{***}, 23–25] summarizes the current evidence for therapies in NASH.

Therapies of potential value for the treatment of nonalcoholic steatohepatitis

Very few large RCTs have been published on which evidence-based treatment for NASH is recommended. Therapies with potential benefit in NASH include thiazolidinediones (TZDs) and vitamin E.

Thiazolidinediones

The most extensively studied and for which the best data are available is for the use of TZDs in the treatment of NASH [4]. Central to their action is their ability to ameliorate insulin resistance and promote fatty acid uptake peripherally [26]; free fatty acids are thus diverted away from the liver towards adipose tissue.

TZDs activate the master adipocyte differentiation regulator, peroxisome proliferator-activated receptor (PPAR) γ , allowing transdifferentiation of preadipocytes into insulin-sensitive, fat-storing adipocytes [27–29]. Interestingly, PPAR γ ligands also attenuate liver fibrosis by suppressing the transdifferentiation of hepatic stellate cells into activated

Table 1. Summary of agents tested in nonalcoholic steatohepatitis

Benefit	Agent	Comment
Potential benefit	Pioglitazone	Improves components of the NAS score [12]. Increased risk of bladder cancer and MI [13,14].
	Vitamin E	Significant improvement in histological lesions [12]. However, may increase all-cause mortality [15].
No clear benefit	Metformin	No effect on histology [16,17].
	Statins	
	Atorvastatin	No histological data, but improves liver enzymes and radiological steatosis [18,19].
	Simvastatin	No effect on histology or liver enzymes [20].
	UDCA	Histological data lacking, four RCTs showed no effect on liver enzymes [21].
	PUFAs	No histological improvement in activity [22 ^{***}], but reduction in steatosis radiologically [23].
Unclear benefit	Angiotensin receptor blockers	Improvements in histology (necroinflammation and fibrosis) but study limited to seven patients [24].
	Pentoxyfylline	Improvement in NASH activity score, but not in fibrosis stage. Study limited to 55 patients [25].

MI, myocardial infarction; NAFLD, nonalcoholic fatty liver disease; NAS, NAFLD activity score; NASH, nonalcoholic steatohepatitis; PUFAs, polyunsaturated fatty acids; RCT, randomized controlled trial; UDCA, ursodeoxycholic acid.

myofibroblasts suggesting an additional direct hepatoprotective effect [30–32]. They also have anti-inflammatory effects [33] and increase circulating adiponectin, an antisteatogenic and insulin-sensitizing adipokine [34,35].

The largest multicentre RCT to date included 247 nondiabetic noncirrhotic patients with biopsy-proven NASH who received pioglitazone (30 mg/day), vitamin E (800 IU/day) or placebo for 96 weeks. Histological improvement that included a reduction of two points in the NAFLD activity score (NAS) with no worsening of fibrosis was the primary endpoint; pioglitazone failed to achieve a statistically significant effect compared with placebo [12]. However, it did significantly improve each individual component of the NAS score (steatosis, lobular inflammation and ballooning). When the analysis was confined to patients with definite steatohepatitis on their index biopsy, pioglitazone achieved the primary endpoint. Several other well conducted trials have shown improvements in steatosis, necroinflammation and ballooning [36–38]; however, to date, no study has shown a definite improvement in fibrosis, which is not surprising given the relatively short follow-up periods in most studies. TZD-mediated effects seem to be abrogated upon treatment discontinuation; at 3 months, alanine transaminase (ALT) and homeostatic model assessment return to baseline. In seven out of nine patients who discontinued medication, recurrent NASH was seen at a 48-week posttherapy biopsy [39].

Unfortunately, side-effects (weight gain [40], bone loss/fracture risk [41], increased risk of myocardial infarction with rosiglitazone [13], increased risk of bladder cancer with pioglitazone [14]) and the possible need for long-term therapy [39] have limited widespread acceptance, with rosiglitazone withdrawn from the market in most countries. Pioglitazone remains available and current guidelines suggest consideration in older patients with biopsy-proven advanced fibrosis that are unable to adopt or maintain lifestyle intervention, with continued metabolic risk factors; caution is required in patients with diabetes or those with heart failure [4,42].

Vitamin E

Apart from targeting aspects of the metabolic syndrome that may have beneficial liver effects, liver-specific therapies have also been investigated in NASH. The role of oxidative stress in disease pathogenesis, in particular, has initiated several studies of antioxidants, primarily vitamin E. Vitamin E consists of eight tocopherols; α -tocopherol is the most active. Its presence in the phospholipid bilayer of cell membranes allows prevention of the nonenzymatic oxidation of cell constituents by free radicals.

Vitamin E may also inhibit profibrotic activity [43,44] and downregulates nuclear factor (NF)- κ B-mediated inflammatory pathways in the liver [45]. Preclinical in-vitro and in-vivo studies have shown that in two fibrosis models, vitamin E can ameliorate liver injury blocking both apoptotic pathways and mitochondrial toxicity [46,47].

The Pioglitazone versus Vitamin E versus Placebo for the Treatment of Nondiabetic Patients with Nonalcoholic Steatohepatitis (PIVENS) trial, described earlier, is the largest and most recent study comparing pioglitazone and vitamin E at 800 IU/day to placebo in nondiabetic, noncirrhotic patients; it reported that vitamin E improved all histological lesions in NAFLD except for fibrosis, and patients taking vitamin E had a greater than two-point improvement on NAS score significantly more often compared with placebo [12]. A pilot study suggested that pioglitazone and vitamin E are superior to pioglitazone alone but the study was not appropriately powered [48]. The positive and encouraging results seen in adult NAFLD may not extrapolate to paediatric NAFLD. The largest trial of vitamin E in paediatric NAFLD, the Treatment of NAFLD in Children (TONIC) trial, randomly assigned children and adolescents to receive vitamin E (800 IU/day) vs. metformin (1 g/day) or placebo for 2 years. Vitamin E significantly improved hepatocyte ballooning but not lobular inflammation, steatosis nor fibrosis. The primary endpoint of reduction in ALT levels, more often than placebo, was not met [16].

The results of these studies with vitamin E need to be balanced against the emerging body of data that vitamin E may increase all-cause mortality: an additional 39 deaths per 10 000 people for those on high-dose (400 IU/day) vitamin E in a dose-dependent manner starting at 150 IU/day, much less than the doses trialled in NASH [15]. Furthermore, vitamin E therapy may be associated with an increase in prostate cancer in men above 50 years old according to a large study of 35 000 patients, and a 20% increased risk of haemorrhagic stroke [49,50]. In light of these risks, the current American Association for the Study of Liver Diseases guidelines recommend that use of vitamin E may be considered in nondiabetic adults with NASH, but not diabetic patients or children [28].

Therapies with no clear benefit

Several well recognized pharmacotherapies have been investigated in NASH; currently, they are not recommended for its treatment.

Metformin

Although metformin initially seemed promising in animal models of NASH [51], no histological

improvement in steatohepatitis has been shown in RCTs in adult and paediatric NASH [16,17]. Low-dose metformin could not mitigate the weight gain associated with rosiglitazone in a recent RCT [52], although its effect seems likely to be through weight loss in the small number of treated patients [53]. As it has no effect on histology, metformin is not currently recommended as a targeted treatment for NAFLD.

Statins

Statins are well recognized in the treatment of dyslipidaemia, but their use as a specific treatment for NAFLD is not well evidenced. Data from the Greek Atorvastatin and Coronary Heart Disease Evaluation study did, however, demonstrate a fall in ALT levels with atorvastatin [19] and the St Francis Heart Study showed a reduction in steatosis radiologically with 20 mg daily of atorvastatin combined with vitamins C and E [18]. There are, however, no histological data currently available to support the use of atorvastatin for NAFLD. Although histological data exist for simvastatin, in a trial of 10 patients with biopsy-proven NASH, there was no statistically significant improvement in serum liver enzymes, hepatic steatosis, necroinflammatory activity or stage of fibrosis within or between treatment and placebo [20]. However, its use to reduce cardiovascular risk in patients with NAFLD is clear and there is no evidence to suggest that patients with NAFLD are at increased risk of statin-related liver injury [54].

Ursodeoxycholic acid

Ursodeoxycholic acid (UDCA), by preventing apoptosis and downregulating inflammatory pathways, is an example of a potential cytoprotective agent investigated in NASH. The largest study to date comparing UDCA and placebo showed an unanticipated improvement in the placebo arm, making the effect of the drug hard to interpret [55]. A Cochrane review of four randomized trials using UDCA showed no significant improvement in liver function tests and histological data were lacking [21]. Until there is evidence of clear histological benefit, UDCA cannot be currently recommended for NASH.

Polyunsaturated fatty acids

Despite preliminary data from small studies suggesting that ω -3 polyunsaturated fatty acid supplementation reduces liver fat content [23], a large trial testing two doses of ethyl-eicosapentaenoic acid did not show any histological efficacy [22¹¹].

Therapies with unclear benefit

Some agents tested in NASH have robust preclinical data, but are yet to be investigated in large RCTs.

Angiotensin receptor blockers

Angiotensin receptor blockers are well established in the treatment of hypertension, a second key component of the metabolic syndrome. Experimental work has clearly shown that angiotensin II promotes survival of hepatic myofibroblasts by activation of I κ B kinase-mediated phosphorylation of NF- κ B subunit RelA [56]. A small pilot study of seven patients with NASH treated with losartan for 48 weeks showed improvements in necroinflammation and fibrosis [24]. Larger studies examining the utility of this agent are ongoing.

Pentoxifylline

Pentoxifylline (PTX) is a tumour necrosis factor- α agonist and reduces production of oxygen-free radicals [57]. Animal models have also suggested an antifibrotic effect together with significantly reducing steatohepatitis [58]. The largest and most recent RCT published in 2011 included 55 patients with NASH receiving PTX or placebo; patients on PTX showed a mean 1.6-point improvement on the NAS score vs. 0.1 point in placebo. Although not significant, there was a slight improvement in fibrosis [25]. Before PTX can be recommended as primary therapy, larger and more compelling data are warranted.

Novel approaches

New therapies with strong experimental evidence are currently being trialled in human NASH, and may provide hope of a targeted pharmacotherapy; these are summarized in Table 2 [59–66].

Caspase inhibition (GS-9450)

Preclinical models have shown that hepatocyte apoptosis is a hallmark of NASH [67], the extent of which correlates with disease severity. In a recent phase-2 placebo-controlled trial, 124 patients with histologically characterized NASH were randomized to once-daily placebo or GS-9450, a selective caspase inhibitor, at varying dosages for 4 weeks. In the highest dose group (40 mg), both ALT and cytokeratin-18 fragment levels improved but only ALT reached significance; GS-9450 was safe and well tolerated [68].

PPAR agonists (GFT-505)

GFT-505 is a dual PPAR α and PPAR δ agonist that in animal models of dietary-induced NASH has shown a reduction in steatosis, inflammation and pro-inflammatory genes; interestingly, GFT-505 has also demonstrated antifibrotic properties, independent of metabolic abnormalities [69¹¹]. Human studies have shown that GFT-505 improves liver function

Table 2. Novel agents currently being tested in, or completed, phase 2 trials*

Agent	Action	Effect on NASH pathogenesis	ClinicalTrials.gov identifier
GS-9450	Caspase inhibition	Prevents apoptosis	NCT00740610 [59]
GFT-505	Dual PPAR α / δ agonist	Hepatic glucose utilization, lipoprotein metabolism and anti-inflammatory effects	NCT01694849 [60]
Obeticholic acid	FXR agonist	Promotes insulin sensitivity, decreases hepatic gluconeogenesis and circulating triglycerides	NCT01265498 [61]
Centriciviroc	CCR2/CCR5 antagonist	Interferes with recruitment of monocytes, macrophages and HSCs upon liver injury	NCT02217475 [62]
Liraglutide	GLP-1 agonist	Induces insulin secretion, reduces glucagon secretion	NCT01237119 [63]
Sitagliptin	DPP-IV inhibitors	Prevents degradation of GLP-1	NCT01963845 [64]
GS-6624 (simtuzumab)	Anti-LOXL-2 antibodies	Inhibits formation and repair of extracellular matrix	NCT01672879 [65]/ NCT01672866 [66]

CCR, C–C chemokine receptor; DPP-IV, dipeptidyl peptidase-4; FXR, farnesoid X receptor; GLP-1, glucagon-like peptide-1; HSC, hepatic stellate cell; LOXL-2, lysyl oxidase-like 2; NASH, nonalcoholic steatohepatitis; PPAR, peroxisome proliferator-activated receptor.

*ClinicalTrials.gov accessed on 28 January 2015.

tests, dyslipidaemia and insulin sensitivity in obese, insulin-resistant patients [70,71]. A phase 2b RCT is now ongoing based on these encouraging results [60].

Farnesoid X receptor agonists (obeticholic acid)

Bile acids act as metabolic signalling molecules, aiding dietary lipid absorption, and are involved in cholesterol homeostasis; they are reabsorbed into the enterohepatic circulation and direct hepatic triglyceride and glucose metabolism. They activate nuclear hormone receptor farnesoid X receptor (FXR) and the G protein-coupled cell surface receptor transmembrane G protein-coupled receptor, which inhibit hepatic de-novo lipogenesis, hepatic gluconeogenesis and glycogenolysis and improve insulin sensitivity. In animal studies, FXR activation has anti-inflammatory actions, partly by inhibiting NF- κ B [72,73]. In-vivo evidence exists for a protective effect of FXR agonists against liver inflammation and fibrosis in the methionine–choline-deficient model of NASH [74]. Thus, obeticholic acid, an FXR agonist, was studied in a small pilot trial of 23 diabetic patients with NAFLD. Patients received 6 weeks of the study drug at either 25 mg or 50 mg daily or placebo. Patients on the study drug lost weight with an associated fall in serum γ -glutamyl transferase and an improvement in the non-invasive Enhanced Liver Fibrosis panel observed more often than in the placebo group [75].

A 72-week trial of 273 patients with NASH randomized to obeticholic acid or placebo has recently reported evidence of significant reductions in histologically defined endpoints including degree of steatosis, grade of inflammation/ballooning degeneration and stage of fibrosis [76^{***}]. These

changes were accompanied by mild weight loss and improved clinical biochemistry parameters, also consistent with reduced liver injury. However, a rise in total cholesterol and a disadvantageous change in high-density lipoprotein/low-density lipoprotein ratio were also observed with obeticholic acid treatment [76^{***}]. Despite this, the agent remains one of the first in which robust, beneficial changes in liver histology have been identified.

Other promising agents with anti-inflammatory, antifibrotic or insulin-sensitizing properties currently in development or undergoing testing in RCTs in NASH include dual C–C chemokine receptor 2/5 antagonists, glucagon-like peptide-1 agonists, dipeptidyl peptidase-4 inhibitors and anti-lysyl oxidase-like 2 antibodies (simtuzumab).

Hepatocellular carcinoma

Obesity and diabetes have been well established as risk factors for HCC [77–79]. Recently, HCC has been linked to NAFLD [80], and there is mounting evidence that the same *PNPLA3* genetic variant (I148M, rs738409) that has long been associated with progressive NAFLD also confers an increased risk of NAFLD-HCC [81,82^{*}]. The prevalence of HCC in cirrhotic NAFLD remains undetermined [83], although steatohepatitis was identified as the underlying aetiology in 24% of patients in a series of HCC surpassing all other causes of chronic liver disease [70] and this trend is set to increase further. In the United Kingdom, a more than 10-fold increase in NAFLD-associated HCC has been observed from 2000 to 2010, with NAFLD-HCC accounting for 34.8% of all HCC cases [84]. It is increasingly recognized that NAFLD is a cause of noncirrhotic HCC. A recent Japanese cross-sectional study analyzed 87 cases of HCC occurring in patients with

histologically characterized steatohepatitis; no established cirrhosis was demonstrated in 43 cases [85]. Most worryingly, HCC has been reported in patients even without steatohepatitis [86]. An analysis of a US insurance claims database found NAFLD was the leading condition associated with HCC, with cirrhosis reported in just 46% of these cases [87].

Adipose tissue expansion and subsequent release of proinflammatory cytokines/adipokines [88,89] and lipotoxicity [90] together promote insulin resistance; hyperinsulinaemia results in increased bioavailability of insulin growth factor-1, which further stimulates cellular proliferation and inhibits apoptosis [91]. Metformin, a biguanide that activates adenosine monophosphate-activated protein kinase and has antiproliferative effects has been shown to inhibit hepatocyte proliferation and induce cell-cycle arrest in hepatoma cell lines [92]. Consequently, targeting insulin resistance with metformin has been investigated in observational and case-control studies of HCC [93,94]. Among patients with type 2 diabetes mellitus, metformin was associated with an estimated 62% reduction in the risk of HCC in a recent meta-analysis (odds ratio, 0.38; 95% confidence interval, 0.24–0.59) [95]. However, the ability of metformin to protect against NASH-associated carcinogenesis is not firmly established as human data are retrospective and do not mitigate against treatment assignment bias.

There is biological plausibility that statins reduce cancer risk via HCC-specific (Myc inactivation)

[96,97] as well as antiproliferative, proapoptotic, anti-angiogenic, immunomodulatory and anti-infective mechanisms [98–100]. A recent meta-analysis examining over 1.4 million patients found results to be heterogeneous [101[†]]. Data from observational studies indicated that statins lowered the risk of HCC in various patient populations; however, no clear benefit was found when only rigorously conducted RCTs were included in the analysis [101[†]].

Bariatric surgery

Surgical weight loss interventions have been investigated in the treatment of NAFLD; the most common procedures tested are laparoscopic adjustable gastric banding and Roux-en-Y gastric bypass. Several uncontrolled studies have reported that bariatric surgery has shown to produce significant weight loss and may be beneficial for the treatment of NAFLD [102,103], but the lack of RCTs precluded this conclusion in a Cochrane review [104]. Nevertheless, a recent study reported that on postbariatric biopsy of 160 patients, steatosis resolved in 75% and steatohepatitis resolved in 90%. Fibrosis of any grade resolved in 53% of patients, with even bridging fibrosis resolved in 29% of patients [105]. The effects of bariatric surgery on steatosis and ballooning appear durable in a 5-year sequential biopsy study, but fibrosis worsened significantly, although more than 95% of patients had a fibrosis score F1 or less at 5 years [106]. Clearly, surgical intervention is not a panacea for all patients with NASH, and more

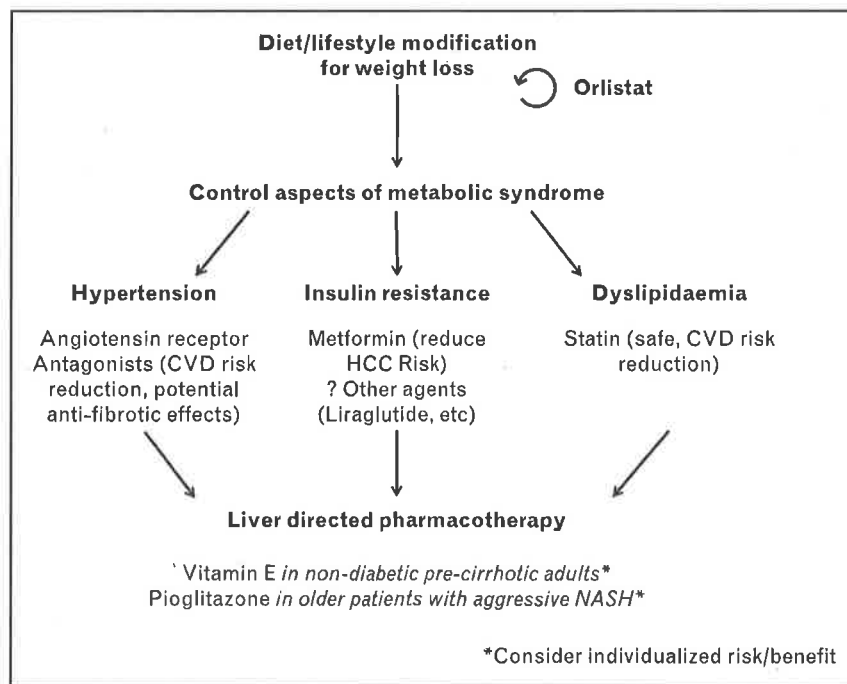


FIGURE 1. Evidence-based schematic for treatment of nonalcoholic steatohepatitis using currently available agents.

robust data from RCTs are needed before recommendations can be made.

CONCLUSION

Despite its prevalence and rising incidence, NAFLD is marked by substantial interpatient variability in prognosis and continues to lack the breadth of therapeutic research and development shared by other causes of chronic liver disease. Although major advances have been made in understanding pathogenesis and also identification of genetic modifiers of liver injury extending beyond simple steatosis including *PNPLA3* and *TM6SF2* [81,107,108[■]], these advances have not yet been fully capitalized upon and so we lack effective pharmacotherapy. Diet and lifestyle modification remain the mainstay of treatment. For patients with NASH and advanced fibrosis, current liver-directed pharmacotherapy with vitamin E and pioglitazone offer some benefits in selected cases. Figure 1 provides a schematic of treatment, once the diagnosis of NASH has been made. However, the beneficial effects of these therapies must be balanced with the potential adverse effects, limiting their widespread use. Coexisting comorbidity must be diagnosed and treated because CVD remains primary cause of death in these patients. The new agents currently in trial provide the first hope of effective, targeted pharmacotherapy in this field.

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Conflicts of interest

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ANSWERING PIVOTAL QUESTIONS IN THE DIAGNOSIS AND TREATMENT OF PRIMARY BILIARY CIRRHOSIS AND NON-ALCOHOLIC STEATOHEPATITIS

Symposium Report from the Intercept Pharmaceuticals, Inc. Supported Satellite Symposium, held at the International Liver Congress™ 2015, the 50th Annual Meeting of the European Association for the Study of the Liver, Vienna, Austria, 22nd–26th April 2015

Chairperson

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Speakers

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MEETING SUMMARY

Professor Trauner introduced the subject of liver disease and its burden within the European Union (EU) and across the globe. Professor Jones summarised the progress made in understanding the pathophysiology of primary biliary cirrhosis (PBC), current unmet needs in the ursodeoxycholic acid (UDCA) era, and novel therapeutic options for PBC treatment. Professor Ratziu discussed the emerging understanding of the complex multisystem pathophysiology of non-alcoholic steatohepatitis (NASH), summarised the available therapeutic targets, and detailed the trials of novel agents currently underway.

Opening Remarks From the Chair

Professor Michael Trauner

Professor Trauner welcomed the audience and thanked the sponsors, Intercept Pharmaceuticals, Inc., for allowing the opportunity to discuss PBC and NASH and answer some key questions in diagnosis and treatment. The audience were invited to engage in the discussion.

PBC and NASH: Serious Liver Diseases with Unmet Needs

Professor Michael Trauner

There is little need to remind an audience of specialists of the importance of liver disease; nevertheless, statistics on its impact on society make for stark reading. Liver disease is a major cause of morbidity and mortality in the EU,

affecting 6% of the population.¹ Chronic disease leads to cirrhosis, hepatocellular carcinoma, and liver transplantation. In the EU, liver cancer mortality stands at 47,000 deaths annually, and more than 5,500 liver transplants are carried out each year.² Overall, liver disease is the fifth-most common cause of mortality in the EU and is implicated in one in six deaths.¹

PBC and NASH stand out amongst the various aetiologies of liver disease, due in part to the recent major advances that have been made in understanding their pathobiology. Increased knowledge of the role played by bile acids in both conditions has helped to develop novel therapeutic targets and has led to improvements in the evaluation and assessment of patients. However, difficulties persist in patient management due to the lack of reliable biomarkers to assist in risk stratification and assessment of patient prognosis in these broad-spectrum diseases. Perhaps the most pressing challenge in the successful treatment of PBC and NASH is the failure of early diagnosis and concomitant lag in treatment, common in both conditions.

PBC Challenges: What is Treatment Success and What Will Emerging Therapies Offer?

Professor David E.J. Jones

There remains significant unmet need in the PBC patient population despite the existence of proven primary therapy in the form of UDCA, as illustrated by the deficit in transplant-free survival in UDCA non-responders compared with age and sex-matched community controls.³ There are a number of possible reasons for the impaired survival of patients treated for PBC: treatments may be used sub-optimally; the effectiveness of current treatments may be overestimated or may be

restricted to a subpopulation of patients, and the distribution of treatments to those in need may be sub-optimal.

In 2008 in the UK, 20% of PBC patients did not receive treatment with UDCA,⁴ and unpublished data indicate that many patients received doses now regarded as insufficient. UDCA also has issues with patient adherence, with barriers including weight gain, nausea, and hair loss. Addressing the above treatment-related issues, using a simple and consistent message underscoring UDCA's effectiveness and the need for all patients to at least receive it at the correct dose, is a logical first step in addressing unmet need.

To identify those UDCA-treated patients with unmet need, patients responding successfully must firstly be characterised. The two principal systems developed to identify treatment response are the Paris and Barcelona criteria (Table 1).^{5,6} These and related criteria (Toronto criteria) have been independently validated using the large UK-PBC cohort, confirming their ability to predict transplant-free survival and consequently the need for their incorporation into routine clinical use.⁷

Recent data from the Global-PBC Group indicate that baseline biomarkers are predictive of treatment outcome. Researchers showed that both elevated alkaline phosphatase (ALP) and bilirubin predict poor clinical outcome.⁸ Thus, both baseline characteristics and treatment response are important to predict event-free survival. This was confirmed by a univariate and multivariate analysis of the UK-PBC cohort, with baseline cirrhosis (as measured by albumin and platelets) and response to UDCA at 12 months (bilirubin, alanine aminotransferase [ALT], and ALP) predictive of transplant-free survival at 15 years. These data are further backed by a 50% treatment failure rate in younger patients in the UK-PBC cohort, despite apparently high overall response rates.⁹

Table 1: UDCA treatment-response criteria.

Paris Criteria	Barcelona Criteria
Bilirubin ≤ 1 mg/dl + AST $\leq 2 \times$ ULN + ALP $\leq 3 \times$ ULN after 1 year of UDCA at 13–15 mg/kg/day	ALP decreased by 40% or normalised after 1 year of UDCA at 13–15 mg/kg/day

UDCA: ursodeoxycholic acid; AST: aspartate aminotransferase; ULN: upper limit of normal; ALP: alkaline phosphatase.

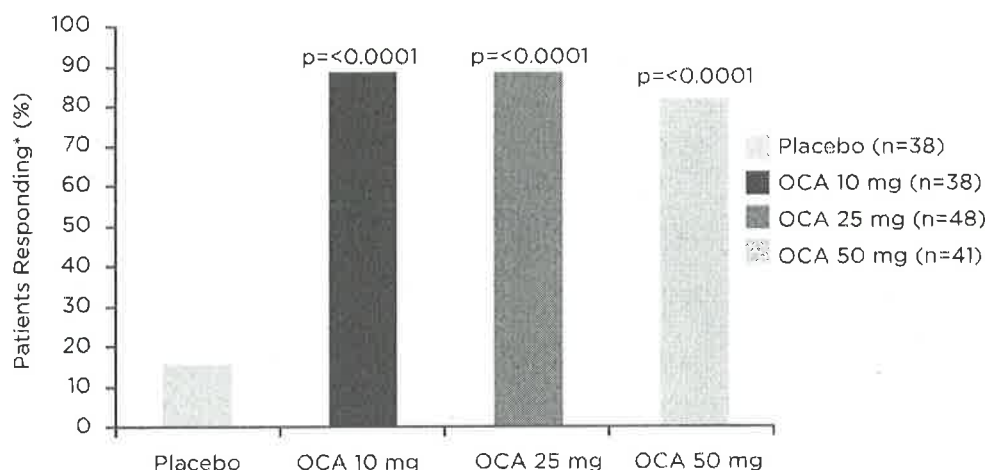


Figure 1: Efficacy of OCA in PBC patients on stable UDCA treatment.

*Primary efficacy endpoint was percentage change in plasma alkaline phosphatase (ALP) from baseline; patients with a placebo-subtracted ALP reduction of $\geq 10\%$ were defined as responders.

OCA: obeticholic acid; PBC: primary biliary cirrhosis; UDCA: ursodeoxycholic acid.

Adapted from Hirschfield GM et al.¹⁴

These younger patients represent a high-risk group in which novel therapies may be most useful.

The above described unmet clinical need necessitates new therapies. There are four elements of the PBC disease process that may offer novel therapeutic targets. The autoimmune response may be addressed through targeted immunosuppression. The secondary cholestatic phase may be amenable to 'second-line' bile acid therapies or manipulation of the microbiota. Biliary epithelial protectant agents may offer a novel route to preserve bile ducts. Finally, those patients who already have fibrosis/cirrhosis may be targeted using antifibrotics. Research into novel therapeutics is complicated by the incomplete picture in terms of biomarkers, difficulties in identifying early stage patients who may respond to immunosuppressant therapies, a lack of clarity regarding therapy-specific response criteria, and the inherent difficulties of trial design caused by a lack of hard endpoints and validated histological measures.

Despite these challenges, it is currently an exciting time for novel PBC therapeutics. Drugs targeting peroxisome proliferator-activated receptor (PPAR)- α (fibrates) and Farnesoid X receptor (FXR) (obeticholic acid [OCA]) systems are joined by norUDCA, which may protect via the creation of a bicarbonate 'umbrella' and has anti-inflammatory and anti-fibrotic effects, and rituximab (RTX),

which targets B cell depletion. A number of other new therapies are also in the early stages of development, including the ileal bile acid absorption blockers A4250 and LUM001, and the immunological agents NI-0801 and ustekinumab.

The fibrates act via PPAR- α agonism, which has been linked to the regulation of bile acid synthesis and detoxification and the modulation of phospholipid secretion, which helps to protect the bile duct epithelium through the formation of micelles.¹⁰ Currently, there is an inadequate number of well-designed trials examining fibrates in PBC. The trials that exist, and associated meta-analyses, have failed to demonstrate clinical efficacy despite biochemical improvements, and have also shown possible safety concerns.¹¹⁻¹³ As a result, despite a logical mechanistic basis, fibrates lack a solid evidence base for efficacy and are associated with possible adverse outcomes in the long term.

The FXR agonist OCA is the most extensively evaluated of the second-line therapies. OCA represents the logical extension of bile acid therapy beyond UDCA, sharing a number of properties (choleretic, anti-apoptotic, and antioxidant effects) as well as a number of additional direct and indirect, FGF19-mediated effects on bile acids. In a recently published Phase II trial (n=165) involving UDCA non-responders, OCA achieved an approximate 90% response rate at all doses tested (Figure 1).

Discontinuation due to pruritus (itch) was an issue in this study,¹⁴ but has been addressed by a dose reduction at Phase III.

Quality of life (QoL) is often the key outcome from the perspective of patients and does not appear to be modified by current treatments. Currently, 35% of PBC patients perceive their QoL as impaired, and almost half feel that their health is worse than it was a year earlier.⁴ B cell depleting agents such as RTX may have a role in reducing fatigue. In summary, the actions needed to improve QoL for PBC patients begin with improving community, patient, and first-physician awareness of the disease and its presentations. Improved physician awareness of the need for therapy with UDCA ($\geq 95\%$) and identification of non-responders must be matched with a systematic approach to management. Built-in triage for high-risk/non-responding patients should migrate these individuals into clinical trials and onto second-line therapies as they become available. In parallel, continued evaluation of second-line therapies and their integration into stratified management pathways is required. Finally, improvement of awareness, assessment, and treatment of symptoms in PBC using systematic approaches and a focus on patients' QoL in addition to the above measures has the potential to dramatically improve the lives of PBC patients.

NASH: Diagnostic Challenges, Therapeutic Targets, and New Paths to Treatment Success

Professor Vlad Ratziu

Recent strides in the understanding of non-alcoholic fatty liver disease (NAFLD) and NASH should soon begin to translate into improved therapeutic options. However, diagnostic challenges still exist, both in terms of disease recognition and risk stratification. Many patients are still underdiagnosed and undermanaged, as in the 61% of patients in retrospectively confirmed cases from a recent database analysis who received no NAFLD care.¹⁵ Beyond recognition of the disease itself, the nature of NAFLD as part of a multi-organ metabolic syndrome must also be recognised. NAFLD is a multi-system disease, and extra-hepatic comorbidities such as Type 2 diabetes (T2D), sleep apnoea, and arterial dyslipidaemia must be addressed. Direct effects of

these comorbidities have been demonstrated, for example, sleep apnoea-related hypoxia modifies the progression of liver fibrosis in NASH. In terms of the liver condition itself, assessment of cofactors of fibrosis in conjunction with disease severity (steatosis/NAFLD or steatohepatitis/NASH), disease stage, and an estimate of prognosis are essential first steps for adequate management.

Identification of patients at risk of progression is a further diagnostic challenge. Recent evidence from serial biopsies suggests that the presence of inflammation and steatosis alone, and not necessarily the full necroinflammatory histology characteristic of NASH, are enough to put patients at risk of progression.¹⁶ Features associated with risk of rapid progression to fibrosis in NAFLD patients include diabetes, metabolic syndrome, magnitude of ALT elevation, and extent of insulin resistance.¹⁷ In NASH patients, risk of progression to severe fibrosis is associated with older age (>45 –50 years) and T2D.^{18,19} There is a small genetic component, with predisposing polymorphisms in *PNPLA3* and *TM6SF2*,^{20,21} as well as associations with obesity, arterial hypertension, hypertriglyceridaemia, insulin resistance, and elevated ALT/aspartate aminotransferase.^{18,19,22} Despite the progress this information represents, further work is needed to identify biomarkers and particularly to create a non-invasive methodology for assessing risk of progression in NAFLD and NASH.

Improvements in the understanding of NASH pathophysiology have led to the identification of new therapeutic targets. NASH pathophysiology appears to derive from metabolic abnormalities, with insulin resistance — particularly in adipose tissue — likely to be the major predisposing disorder. Free fatty acids, chemokines, and insulin drive further metabolic dysregulation as well as directly causing inflammation and cell death, leading ultimately to fibrogenesis and progression towards cirrhosis. This complex and interconnected pathophysiology results in numerous drug targets but also a need to target multiple pathways to reduce fibrogenesis in the long term.

Foremost amongst the novel therapies targeting NASH are FXR agonists, such as OCA. The SCD1 inhibitor aramchol and the PPAR agonist GFT505 work by reducing liver fat, while the dual CCR2 and CCR5 antagonist cenicriviroc targets inflammation and may also have antifibrotic effects.

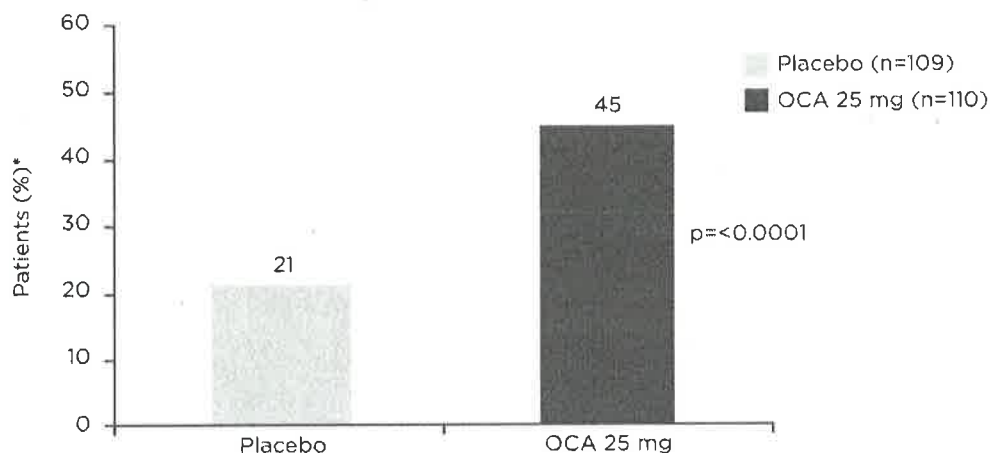


Figure 2: Patients treated with OCA achieving primary outcome measure.

*≥2-point improvement in NAS score without worsening of fibrosis.

OCA: obeticholic acid; NAS: NASH activity score.

Adapted from Neuschwander-Tetri et al.³¹

Other drugs target fibrogenesis by blocking collagen cross-linking (anti-lysyl oxidase-like 2 [LOXL2] monoclonal antibody, simtuzumab [SIM]) or by inhibiting the fibrosis-related protein galectin-3. The urgent need for effective therapies for NASH is recognised by the FDA, as illustrated by the granting of breakthrough status to OCA and fast-track status of the majority of the other novel compounds mentioned.²³⁻²⁷

The main mode of action of FXR-agonist therapies, such as OCA, in NASH is through direct cytochrome-modulated blockade of conversion of cholesterol in bile acids. However, as noted above, indirect effects via FGF19 are also present, which may act on metabolic pathways, improving glucose tolerance and insulin sensitivity and reducing lipogenesis and hepatic fat. Direct antifibrotic properties derived from blocking activation of quiescent hepatic stellate cells may also play a role.²⁸⁻³⁰ Data are available from a Phase IIb 72-week randomised, double-blind, placebo-controlled trial of OCA 25 mg/day (n=110) versus placebo (n=109) with both clinical and histological endpoints.³¹ NASH patients with active disease were eligible. There was a striking difference in the number of patients achieving the primary histological outcome measure (improvement in NASH activity score [NAS] ≥2) between placebo and OCA-treated patients (Figure 2).

There were across-the-board significant improvements in every histological feature that defines NASH (lobular inflammation, steatosis,

hepatocellular ballooning, and fibrosis). This represents the first human demonstration of antifibrotic efficacy in NASH, particularly noteworthy given that the trial was not powered for this outcome.³¹ Adverse event data showed mild-to-moderate effects in general. As with the above PBC data, pruritis was an issue;³¹ however, the PBC data also suggest that it may be addressed via dose adjustment.

As mentioned, the conjugated bile acid-saturated fatty acid aramchol modulates the amount of fat in the liver. It acts via two pathways: inhibition of fatty acid metabolism via blockade of SCD1 enzyme activity, and activation of cholesterol efflux by stimulating the cholesterol pump ABCA1.³²⁻³⁴ Results from a small (n=57) Phase IIa trial indicate that aramchol dose-dependently reduces liver fat, as measured by non-invasive magnetic resonance spectroscopy. This result is currently being confirmed in a population of NASH patients with active disease and metabolic syndrome in the Phase IIb ARREST trial (n=240). In addition to steatosis, NASH resolution, reduced NAS score, and metabolic improvements will be assessed.

Cenicriviroc is a dual CCR2 and CCR5 antagonist that has shown potential for antifibrotic activity.³⁵ These two cytokines have overlapping proinflammatory and profibrotic properties, aiding the chemotaxis of inflammatory cells and activating profibrotic stellate cells.³⁶ The large international Phase II CENTAUR trial³⁷ (n=252) of cenicriviroc includes patients that have well-defined NASH

either with active disease or progression risk factors. The primary outcome is improvement of NAS score with no worsening of fibrosis, with the main secondary outcome being resolution of NASH with no worsening of fibrosis, which is likely to be important for approval as a NASH therapeutic.

As a selective dual PPAR- α and PPAR- δ agonist with no PPAR- γ activity, GFT505 combines liver-specific (PPAR- α) and multi-organ anti-inflammatory and fat-reducing activity (PPAR- δ).³⁸ Phase II trials have demonstrated improved lipid metabolism and insulin sensitivity in diabetic and pre-diabetic patients, and animal data suggest the presence of anti-inflammatory and antifibrotic properties in NASH.^{39,40} The 1-year Phase IIb GOLDEN trial⁴¹ (n=270) of GFT505 is highly anticipated, with preliminary results suggesting that the primary endpoint of NASH resolution with no worsening of fibrosis has been met.

SIM directly targets fibrosis through highly specific inhibition of LOXL2, the enzyme that promotes the cross-linking of collagen, which is key to the fibrotic process. LOXL2 levels may correlate with clinically relevant NASH endpoints, and blockade of collagen cross-linking has been demonstrated in other conditions.^{29,42} Two large 240-week Phase IIb trials are currently underway comparing two doses of SIM (75 mg and 125 mg) with placebo in either cirrhotic (n=259) or non-cirrhotic (n=222) patients. In the cirrhotic patients, the drug is administered intravenously every 2 weeks with a liver biopsy after 1 year, and endpoints are based on hepatic venous pressure gradient and event-free survival. In the non-cirrhotic trial, participants with NASH and bridging fibrosis receive a weekly subcutaneous injection; the primary endpoint is fibrosis and event-free survival (assessed as time-to-progression to cirrhosis).⁴³

Challenges remain in the NASH therapeutic pipeline. The multiple pathogenic mechanisms of NASH require therapies that target more than one pathway to achieve histological efficacy. Improved animal models and non-invasive outcomes for proof-of-principle trials are needed to speed up development. The lack of surrogates for hard outcomes, in particular non-histological outcomes, and response on therapy are also issues; however, an effective primary therapy will be required before this can be addressed. Nevertheless,

there has been tremendous progress in the field recently. Firstly, the medical need is now being recognised for NASH as a standalone condition related to, but not subsumed by, metabolic disorder. NASH is accepted as an indication for therapy and now has operational pathological definitions. Achievable surrogate endpoints have been set and the regulatory path for approving drugs in NASH is clear, with regulatory bodies behind the push for new therapies.

In conclusion, it is essential to assess liver injury in those with metabolic risk factors such as diabetes or obesity. NAFLD is a cause of liver cirrhosis and primary liver cancer, and prognosis is dependent on the fibrosis stage and also the presence of steatohepatitis (NASH), which ultimately drives fibrosis. There is a need to develop pharmacological agents that target NASH and a number of these are now being tested in large Phase IIb trials, with OCA soon moving into Phase III trials. Once we have demonstrated the efficacy of these drugs, tailoring of therapy to individuals and integrative approaches with diet and lifestyle will be the key concerns.

Concluding Remarks

Professor Michael Trauner

In summary, a significant proportion of patients with PBC have insufficient response to available treatment and require novel therapies. New strategies based around second-line bile acid therapies, particularly OCA, appear to be yielding results, while new immunological approaches targeting symptoms may help address key patient concerns such as fatigue. NASH remains an under-recognised liver disease in clinical practice. New non-invasive detection methods to track progression and measure therapeutic efficacy are needed, although some progress has been made in tracking fibrosis and the inflammatory component of the condition. Treatment of metabolic comorbidities may have a beneficial impact on liver disease but there is an urgent need for novel therapies beyond lifestyle modification. A number of new therapies are at Stage II of testing, including second-line bile acids and others targeting metabolic aspects of the disease alongside inflammation and fibrosis.

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Nonalcoholic Fatty Liver Disease (NAFLD), a Manifestation of the Metabolic Syndrome: New Perspectives on the Nutritional Therapy

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Abstract

Nonalcoholic fatty liver disease (NAFLD) is a multifactorial hepatic disease that develops through complex mechanisms that may be strongly influenced by dietary composition. NAFLD treatment is based on multidisciplinary intervention, which includes nutritional aspects. The objective of this review was to elucidate the influence and role of dietary composition, including fatty acid types, antioxidant nutrients, pre and probiotics and vitamin D in the nutritional treatment and prevention of NAFLD. Increased intake of Monounsaturated fatty acids (MUFAs) and omega-3 polyunsaturated fatty acid (PUFA), particularly as replacements for saturated fat and in a higher proportion than carbohydrates, is beneficial to NAFLD patients, improving insulin resistance; increasing plasma levels of adiponectin and its synthesis by the adipose tissue; and restoring the expression of hepatic peroxisome proliferator-activated receptor alpha (PPAR- α), which in turn reduces cholesterol levels and triacylglycerol accumulation. n-3 PUFAs can reduce lipotoxicity caused by excessive saturated and trans fatty acid ingestion and exert a protective role in inflammatory pathways, promoting resolvins and protectins. Several mechanisms linking gut flora to NAFLD have been proposed, such as inflammation and energy extraction. Studies are often designed to explore the beneficial effects of probiotics, prebiotics and vitamin D in these pathways. The results of this review reveal that the strong positive influence bioactive compounds have on these inflammatory processes must be considered when developing treatment and prevention plans for NAFLD patients.

VitD

Keywords: NAFLD; Nutrition; Fatty acids; Intestinal microbiota; Vitamin D

Introduction

Nonalcoholic fatty liver disease (NAFLD) is defined as the abnormal accumulation of lipids, primarily in the form of triglycerides in individuals who do not consume significant amounts of alcohol (≤ 20 g ethanol/d) [1]. NAFLD is characterized by a spectrum of disease varying from simple steatosis through to steatohepatitis with fibrosis and scarring, which can lead to cirrhosis [2].

The prevalence of individuals diagnosed with NAFLD varies from 10-39% of the world population. The disease can be associated with other co-morbidities and affects 50% of diabetics, 57 to 74% of obese people, and 90% of morbidly obese people. In the pediatric population, NAFLD affects 2.6% of eutrophic children and up to 60% of obese children and adolescents [3-5]. NAFLD is currently recognized as a clinically emergent problem among obese patients [6,7].

NAFLD is a multifactorial disease and is the hepatic manifestation of metabolic syndrome. The pathogenesis of NAFLD involves complex mechanisms and has been extensively discussed in the literature. Insulin resistance (IR), inflammatory states, nutrients, genetic factors and lifestyle all play key roles in its development [8]. The central mechanism responsible for NAFLD is insulin resistance, which causes an influx of free fatty acids into hepatocytes, elevates de novo hepatic lipogenesis that can exceed the rate of β -oxidation of fatty acids and causes very low density lipoprotein (VLDL) exportation, ultimately resulting in hepatic fat accumulation [7]. This disequilibrium, caused by hepatic lipid influx, can induce reactive oxygen species (ROS) production, which increases oxidative stress and activates stellar hepatic cells. Molecular lipotoxicity can occur once the influx of free fatty acids activates complex intracellular pathways, including the c-jun N-terminal Kinase enzyme and the Toll-like 4 receptor (TLR-4). Moreover, increased lipid peroxidation leads to the generation of ROS, which are toxic mediators that ultimately promote mitochondrial dysfunction in hepatocytes [9,10].

Visceral adipose tissue accumulation, which contributes to inflammatory pathways and the development of peripheral insulin resistance, has also been explored as a potential mechanism for

developing NAFLD [11]. Inflammatory cells, such as macrophages, infiltrate visceral adipose tissue, which increases inflammatory adipokine secretion, leptin, interleukin 6 (IL-6), tumor necrosis factor alpha (TNF- α) and angiotensinogen as well as reduces adiponectin production [12]. When the tissue becomes insulin-resistant, it induces major lipolytic activity and increases the levels of free fatty acid influx into the portal vein, potentially causing hepatotoxicity. Small intestinal bacterial overgrowth (SIBO) may also promote the progression of NAFLD into nonalcoholic steatohepatitis by enhancing intestinal permeability and favoring absorption of endotoxins with pro-inflammatory and pro-fibrogenetic effects on the liver [13]. It is worthwhile to consider that the microbiota and nutrients involved in intestinal health can inform strategies for NAFLD treatment.

Several studies have reported the important role that dietary composition plays in the development and progression of NAFLD [14-16]. A high-fat diet converts the pathology from bland NASH with fibrosis, which leads to cirrhosis in humans [17]. It has been proposed that the levels and types of fatty acid composition, especially saturated and trans fatty acids, promote the accumulation of free fatty acids (FFA) in hepatocytes, which causes apoptosis by diverse inflammatory pathways. These may include microbiota modification, insulin resistance, and ROS-induced stress that affect the mitochondrial membranes, endoplasmic reticulum and lysosomes (see review Estadella et al., [10]).

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Lipid peroxidation increases the levels of reactive oxygen species, which may be partially responsible for hepatocyte dysfunction. Antioxidant nutrients reportedly exert an important role in reducing ROS production [18]. Moreover, mono and polyunsaturated fatty acids can play a protective role in NAFLD development, mainly because n-3 PUFAs (polyunsaturated fatty acids) have many beneficial effects on most metabolic syndrome features and have anti-inflammatory properties [19].

The objective of this review was to elucidate the influence and role of dietary composition, including the type of consumed fatty acids, antioxidant nutrients, pre and probiotics and vitamin D in the nutritional treatment and prevention of NAFLD.

NAFLD and Lipids

Fatty acids are an essential constituent of the cell membrane, as they modulate the activity of membrane-bound transporters and enzymes, modify membrane fluidity, serve as intracellular messengers and alter intracellular functions. One animal study reported that dietary lipids can change both the chemical composition and lateral organization of rat hepatocyte plasma membranes [20].

In humans, dietary fat intake may play a critical role in the development of NAFLD. It is now clear that both the levels of lipids introduced and the types of fatty acids ingested affect the development of the disease [21].

The lipid metabolic perturbations in NAFLD are complex and may be more fully explored. The evaluation of the amounts and quality of different lipid classes, comparison of fatty acid distribution and comprehension of the functions fatty acids have in different NAFLD mechanisms can be an important tool for NAFLD treatment [21]. Recently, lipotoxicity and plasma lipidome have emerged as novel targets for potential therapeutic strategies [21,22]. The possible mechanisms by which lipids affect NAFLD treatment will be further explored in the present review.

NAFLD and mono and polyunsaturated fatty acids

Diet composition may directly and indirectly influence the NAFLD pathways. Dyslipidemia is considered a risk factor for NAFLD development [23]. Several studies have demonstrated that a high intake of monounsaturated fat improves postprandial glucose, decreases oxidized low density lipoprotein (LDL), LDL cholesterol, and triacylglycerol concentrations, particularly for a diet in which monounsaturated fats replace both saturated fat and high levels of carbohydrates [8,24-26].

A recent study has demonstrated that in only 6 weeks, an olive-oil rich diet, the Mediterranean diet, which contains high levels of monounsaturated fatty acids, promotes a decrease in hepatic steatosis accompanied by an improvement in peripheral insulin sensitivity and a reduction in circulating insulin concentrations [27]. These authors also suggested that an increased intake of monounsaturated fatty acids (MUFAs) and omega-3 PUFAs, particularly as a replacement for saturated fat and as a higher proportion of the diet than carbohydrate intake, is beneficial for NAFLD patients. Some studies suggest that insulin resistance may be accompanied by a change in the composition of fatty acids in the blood and tissues, with deficiency of omega 3polyunsaturated fatty acids. Moreover, there have been promising results in animal models and humans with the use of omega 3 in NAFLD [28,29].

The beneficial effects of supplementing with omega 3 can be partially explained by the increase in the plasma levels of adiponectin as

well as the increased synthesis of adiponectin in the adipose tissue and the restored expression of hepatic peroxisome proliferator-activated receptor alpha (PPAR- α), promoting a reduction in the cholesterol levels and accumulation of triacylglycerol [30].

Several authors have shown that n-3 PUFA may have beneficial effects in preventing the complications of lipotoxicity that are primarily caused by excess saturated and trans fatty acids. PUFA dietary intake has beneficial effects on intra-hepatic fat accumulation in patients with NAFLD [19,21,31]. In a study on a pediatric NAFLD population, n3-docosahexaenoic acid treatment for 6 months improved the fatty liver and insulin sensitivity [29].

Omega 3 (n-3) PUFAs, especially eicosapentaenoic acid (C20:5n3, EPA) and docosahexaenoic acid (C22:6n3, DHA), exert protective roles in the inflammatory pathways involved in NAFLD development and progression [19].

The omega-3 (n-3) fatty acids are essential, polyunsaturated fatty acids (PUFAs) that cannot be synthesized in vivo. Instead, they are consumed in the diet, especially from fish oil, flaxseed and some nuts. These fatty acids, which are derived from α -linolenic acid and mainly occur as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), are anti-inflammatory. The other key group of PUFAs, n-6 fatty acids, is predominantly found in grain and derived from linolenic acid; arachidonic acid is proinflammatory and prothrombotic [28].

Omega-6 (n-6) and omega-3 (n-3) fatty acids (PUFAs) are competitively metabolized by the same pathway. In this way, the ratio of n-6 to n-3 should be approximately 3:1; however, there is no consensus on the adequate ratio for achieving beneficial effects in NAFLD. Although this ratio has not been established, it is strong confirmation that a low total level of n-3 is found in NAFLD and is associated with steatosis, increased oxidative stress and progression of the disease [28].

Briefly, the n-6 and n-3 fatty acids compete for enzymes involved in chain desaturation and elongation reactions. Although these enzymes have higher affinity for the n-3 family, the conversion of α -linolenic acid in PUFA-LC is strongly influenced by the levels of linoleic acid in the diet. Thus, the ratio between the daily intake of food sources of fatty acids n-6 and n-3 is important in human nutrition [32-34].

Studies have revealed a significant reduction in the mortality rate of patients with cardiovascular disease, inflammation, and symptoms resulting from asthma when the ratio of linoleic/ α -linoleic in the diet is 4:1; the symptoms were intensified for a ratio of 10:1 [32-34].

Moreover, there was a study in which the exposure of human hepatocytes to different mixtures of linoleic and α -linoleic affected the transcription levels of a selection of genes encoding regulatory proteins that are involved in several stages of fatty acid metabolism. This effect strongly depends on the ratio of n6/n3 fatty acids, revealing the importance of ingesting not only appropriate levels of fatty acids but also an appropriate ratio of n6 and n3 fatty acids [35].

Arachidonic acid, di-homo- γ -linoleic acid (20:3 n-6), and eicosapentaenoic acid (20:5 n-3) are precursors of series 1, 2 and 3 prostanoids and series 4, 5 and 6 leukotrienes, respectively. Cyclooxygenase (COX), which converts these free fatty acids in cyclic endoperoxides, produces prostaglandins and thromboxane, the prostanoids. Lipoxygenase (LOX), which is also linked with the production of lipoxins 1,9, contributes to the production of leukotrienes [36].

Increased levels of fish oil decrease the levels of arachidonic acid in the inflammatory cell membranes, resulting in less substrate availability

for the synthesis of eicosanoids that are derived from arachidonic acid. Additionally, EPA inhibits arachidonic acid release from phospholipids, which is mediated by phospholipase A2, and competitively inhibits the COX-mediated oxygenation of arachidonic acid. Thus, n-3 fatty acids have important anti-inflammatory properties, including decreasing the production of series 2 prostaglandins and thromboxanes [37].

The excessive production of series 2 prostanoids may be related to immune disorders, cardiovascular disease, and inflammation. Thus, people are encouraged to increase their intake of n-3 fatty acids to increase the production of series 3 prostanoids [36].

Alterations in the liver long chain polyunsaturated fatty acids (LCPUFA) status in NAFLD are characterized by significant depletion of the n-3 LCPUFA content and an increased in the n-6/n-3 LCPUFA ratio, which can favor the development of NAFLD [38].

Studies investigating whether dietary n-3 LCPUFA supplementation triggers an antioxidant response that prevents liver steatosis have demonstrated that supplementation results in a reduction in the hepatic lipid content; increased insulin sensitivity; and decreased levels of serum transaminase, TNF- α , soluble TNF receptor 1 and 2, and oxidative stress markers, with improvement of hepatic steatosis. These outcomes are mediated by either n-3 LCPUFA derived resolvins and protectins and/or direct PPAR- α -dependent factor nuclear kappa B (NF- κ B) and inhibition of the activator protein 1 (AP-1) [39-41].

Omega-3 fatty acids are key regulators of the transcription of hepatic genes, such as PPAR α and sterol regulatory element binding protein-1 (SREBP-1). PPAR α reduces plasma lipids and increases mitochondrial beta oxidation, and omega-3 fatty acids activate this transcription factor, improving the expression of genes associated with fatty acid and lipid metabolism [42]. The SREBP-1 influences the genes involved in fatty acid and cholesterol synthesis, reducing endogenous lipid production and improving peripheral insulin sensitivity [28]. Moreover, the levels of omega 3 fatty acids in the diet can substantially influence the stimulation of adiponectin, an anti-inflammatory adipokine, gene expression [43].

EPA and DHA promote decreases in the levels of anti-inflammatory and inflammation resolving resolvins and protectins [37]. The resolvins and protectins are new families of locally acting mediators that are derived from essential fatty acids. The synthesis of resolvins and protectins involves the COX and LOX pathways, and their biological effects of have been widely evaluated in cell culture and animal models as anti-inflammatory and inflammation resolving; for example, they inhibit the transendothelial migration of neutrophils, preventing the infiltration of neutrophils into sites of inflammation and inhibiting IL-1 β production. Furthermore, protectin D1 specifically inhibits the synthesis of TNF- α and IL-1 β [37].

Several studies have confirmed that n-3 fatty acids are involved in the regulation of hepatic gene expression, cell membrane composition, insulin sensitivity, anti-inflammatory effects, and the reduction of TNF- α levels, decreasing liver steatosis [19,21,28,31,44,45].

NAFLD, intestinal health and inflammatory pathways

Multi-targeted therapy could optimally treat NAFLD patients. Recently, interesting nutritional strategies like the use of dietary supplements, such as probiotics and long chain omega-3 polyunsaturated fatty acids, have been adopted in NAFLD treatment [28,46,47].

Among the pathogenic factors leading to NAFLD, crosstalk between the gut and liver is critical [48]. Currently, specific nutrients that are capable of increasing the intestinal permeability to bacterial

endotoxins promote the inflammatory response of liver cells, leading to a profibrogenic state [49]. A recent study utilizing animal models [50] demonstrated that restoring the gut microflora is critical to protecting the liver from fat accumulation, which reinforces that nutrients that act on inflammatory pathways to improve gut health and promote adequate microbiota colonization, such as pro and prebiotics and vitamin D, should be considered in NAFLD treatment.

NAFLD: Probiotics and Prebiotics

Microbiota

The human gut contains substantial microorganisms, including bacteria, archaea, viruses, and some unicellular eukaryotes, called microbiota, which may provide energy, nutrients, and immunological protection that benefit the host [51].

The intestinal flora is important to normal gut function and maintenance of health, and the dietary composition can influence the intestinal colonization. In healthy adults, 80% of the identified fecal microbiota can be classified into the following three dominant phyla: Bacteroidetes, Firmicutes and Actinobacteria. The Firmicutes to Bacteroidetes ratio is important in the human gut microbiota composition [52]. Studies have demonstrated that human obesity is associated with low levels of intestinal Bacteroidetes and high levels of Firmicutes, favoring the development of metabolic syndrome and NAFLD [53,54].

Several mechanisms have been proposed that link the gut flora to obesity; for example, the gut microbiota increase energy extraction from indigestible dietary polysaccharides and elevate plasma lipopolysaccharide levels, resulting in chronic low-grade inflammation. Additionally, the gut microbiota in regulate the host genes that control metabolic processes [55,56].

Gut Microbiota: Inflammation and Energy Extraction

Inflammatory process

Germ-free mice are protected from obesity and metabolic syndrome. There may be complex mechanisms for the potential increased energy harvest from the diet through dietary-induced or genetically induced obese microbiota [57]. However, experimental protocols that colonize germ-free mice with selective human flora are essential for investigating the influence of diets and other confounding factors on the microbiota and their consequent implications for the host metabolism [58].

The quality of the diet influences the gut microbiota composition. Diet explains 57% of the bacterial variation in the gut while genetic background only accounts for 12% of the variation in animals, suggesting the importance of diet in determining the composition of the gut microbiota [59].

Cani et al. [55] found that mice treated with a high-fat diet have a significant change in the composition of their dominant bacterial populations within the gut microflora, including a decrease in the number of Bifidobacteria, Eubacterium rectal-Clostridium coccoides group and Bacteroides, favoring an increase in the gram-negative to gram-positive ratio. This change in the gut microflora composition was associated with a significant increase in plasma lipopolysaccharide (LPS) levels accompanied by an increase in the intestinal permeability and a reduction in the expression of genes coding for tight junction proteins [60,61]. These studies demonstrate the possible pathways that are responsible for the gut microbiota in metabolic endotoxemia, inflammation, obesity, liver hepatic triacylglycerol accumulation, insulin resistance and type 2 diabetes [62,63].

Increasing evidence suggests the gut microbiota in humans controls obesity and visceral fat storage [64]. Small intestinal bacterial overgrowth (SIBO), a common condition in obese individuals, is mainly stimulated by slowing of the oro-coecal transit time, may promote NAFLD progression to nonalcoholic steatohepatitis by enhancing intestinal permeability and favoring absorption of endotoxins with pro-inflammatory and pro-fibrogenetic effects on the liver [65].

In previous clinical studies performed by our research group, we found a positive correlation between the calories derived from SFA intake and visceral fat in NAFLD patients [66,67]. Moreover, we recently demonstrated a positive correlation between the plasma endotoxin concentration and pro-inflammatory cytokines, especially IL-6, and insulin resistance in obese adolescents. After long-term (one year) interdisciplinary therapy, endotoxemia, pro-inflammatory status and insulin resistance were decreased [68]. These results demonstrate the efficiency of lifestyle changes (i.e., nutritional modification) in reducing the pro-inflammatory state of obese individuals [69].

An increase in serum LPS has been associated with the proinflammatory state, development of insulin resistance and type 2 diabetes. LPS stimulates Toll-like receptors in the cell membranes, which activate specific kinases and lead to insulin resistance [68].

These pathways also activate NF- κ B, which results in the expression of inflammatory genes. Similar to LPS, saturated fatty acids are also recognized by membrane receptors that trigger proinflammatory signaling pathways [56,62,63,68].

Energy Extraction: Metabolic Processes

The bacterial metabolites, TMAO (trimethylamine-N-oxide) and SCFA (short chain fatty acids) are markers of risk for disease and have varied effects on metabolism [51].

TMAO is a phospholipid that is integral to cell membranes and is present in foods with higher fat contents that are associated with the atheroprogession process, which is influenced by the microbiota composition [51]. This phospholipid is a new biomarker for developing CVD as well as a novel biomarker for food choice behavior. In fact, NAFLD has recently been associated with the pro atherogenic state [70].

The SCFAs produced by microbiota fermentation are acetate, propionate and butyrate, which are used as an energy source for colonocytes, and the overproduction of SCFAs could increase the liver lipogenesis because they are a lipogenic substrate [71]. Indeed, SCFAs are affected by the gut microbiota composition and influence the regulation of blood lipids as well as affect pathways related to food intake and lipid metabolism. The intestinal microbiota break down indigestible polysaccharides (i.e., fiber) to short-chain fatty acids (SCFAs) providing 80 to 200 kcal per day or approximately 4–10% of the daily energy intake in normal adults [72].

The mechanisms supporting that the absence of adequate gut microbiota is associated with the development of obesity and NAFLD are related, in part, to the effect of healthy microbiota on the reduction of hepatic de novo lipogenesis as well as to the inhibition of triacylglycerol storage in the white adipose tissue. The latter effect is thought to be caused by an excessive production of FIAF (fasting-induced adipocyte factor) or angiopoietin-like protein 4 (ANGPTL4) in the intestines of the germ-free mice [73].

FIAF is a circulating lipoprotein lipase inhibitor produced by the intestines, liver and adipose tissue. FIAF inhibits lipoprotein lipase (LPL), blocking the disassociation of fatty acids from triacylglycerols

for uptake into tissues as well as upregulating fatty acid oxidation and uncoupling proteins, potentially reducing the fat storage in germ-free mice [74]. FIAF also plays a role in the metabolic adaptation to fasting via PPAR activation [75].

Several systems in people can be affected by bacterial components and metabolites of the gut microbiota detecting, especially in the case of the development of metabolic diseases [51]. The gut microbiota can directly and indirectly affect the host's health through bacterial components and metabolites.

Probiotic Effects

The probiotics promote gastrointestinal health and beneficial consequences for the liver.

In colonic epithelia, probiotics stimulate mucin production and improve the self-protecting properties of the intestinal epithelium by stimulating tight junctions, which competitively exclude microbial pathogens. Indeed, the tight junction proteins remain intact and, thereby, prevent both the uptake of macromolecules and translocation of viable organisms to mesenteric lymph nodes and the liver [76].

Moreover, the integrity of the intestinal barrier decreases the LPS exposure and proinflammatory signaling, improving the anti-inflammatory cytokines, such as interleukin-10, which results in less vulnerability to hepatotoxins and limits intestinal bacteria overgrowth (IBO) and LPS production [76]. Altogether, probiotic supplementation is an important tool for treating NAFLD patients.

Corroborating these findings, studies have demonstrated that gut microbiota manipulation with probiotics in rodents with fatty livers reduces intestinal inflammation and improves the function of the epithelial barrier [60,61]. Hence, probiotics could be used to treat NAFLD human patients [13].

Loguercio and colleagues [77] showed that probiotics may reduce NAFLD liver injury and improve liver function tests. Another study found that treatment with 500 million *Lactobacillus bulgaricus* and *Streptococcus thermophilus*/day in adults with biopsyproven NAFLD promotes a significant reduction of the levels of liver transaminases [78].

These data suggest that the diet composition has an important role on the development and treatment of NAFLD [67]. Additionally, it is essential to consider the use of probiotics to treat and prevent NAFLD [62].

To further elucidate the role of the gut microbiota and gut-liver-axis both in causing or worsening obesity itself and/or related complications including NAFLD and NASH, robust, well-designed studies should be performed. These studies should focus on the mechanisms involved in the possible imbalances of the numerous metabolic, toxic, and immunological actors participating in the gut-liver-axis. The probiotic-mediated gut microbiota modulation is a promising tool for treating NAFLD, NASH, and obesity due to the safety, tolerability and efficacy of this treatment method [78,79].

Using probiotics as an intervention for intestinal microbiota can modulate the expression of nuclear receptors, improving insulin resistance in the liver and adipose tissue as well as protecting against the development of NAFLD. Randomized placebo controlled trials on the use of probiotics in NAFLD are ongoing in humans [46-48].

NAFLD and Prebiotics

Prebiotics are beneficial, non-digestible food ingredients that

affect the host by selectively stimulating growth and/or modifying the metabolic activity of select intestinal bacteria [80]. Various fermented ingredients are classified as prebiotics, including inulin-type fructans and galactans [81].

The main health effects of prebiotics are due to three principal mechanisms that include selective modulation of the gut microbiota to reduce inflammation; improvement of glucoregulation and modification of lipid metabolism including reduced de novo fatty acid synthesis and SCFA production; and reduction of body fat [81,82].

Modulation of the gut microbiota is linked to improvement in the glucose, energy intake, insulin, satiety hormones, hepatic cholesterol, and triacylglycerol accumulation. Prebiotics have been reported as a potential therapeutic approach for reducing the risk of obesity and altering the composition of the gut microbiota (Figure 1) [78].

Prebiotic fibers have a bifidogenic effect and are associated with reduced LPS levels. As stated before, LPS, also known as lipoglycans, are large molecules consisting of a lipid and a polysaccharide joined by a covalent bond; LPS are found in the outer membrane of gram-negative bacteria, act as endotoxins and elicit strong immune responses in animals [83].

Jumpertz et al. [84] reported that the stool energy in proportion to the ingested calories is positively correlated with the abundance of the

phylum Bacteroidetes and negatively correlated with the abundance of the phylum Firmicutes in the feces of people.

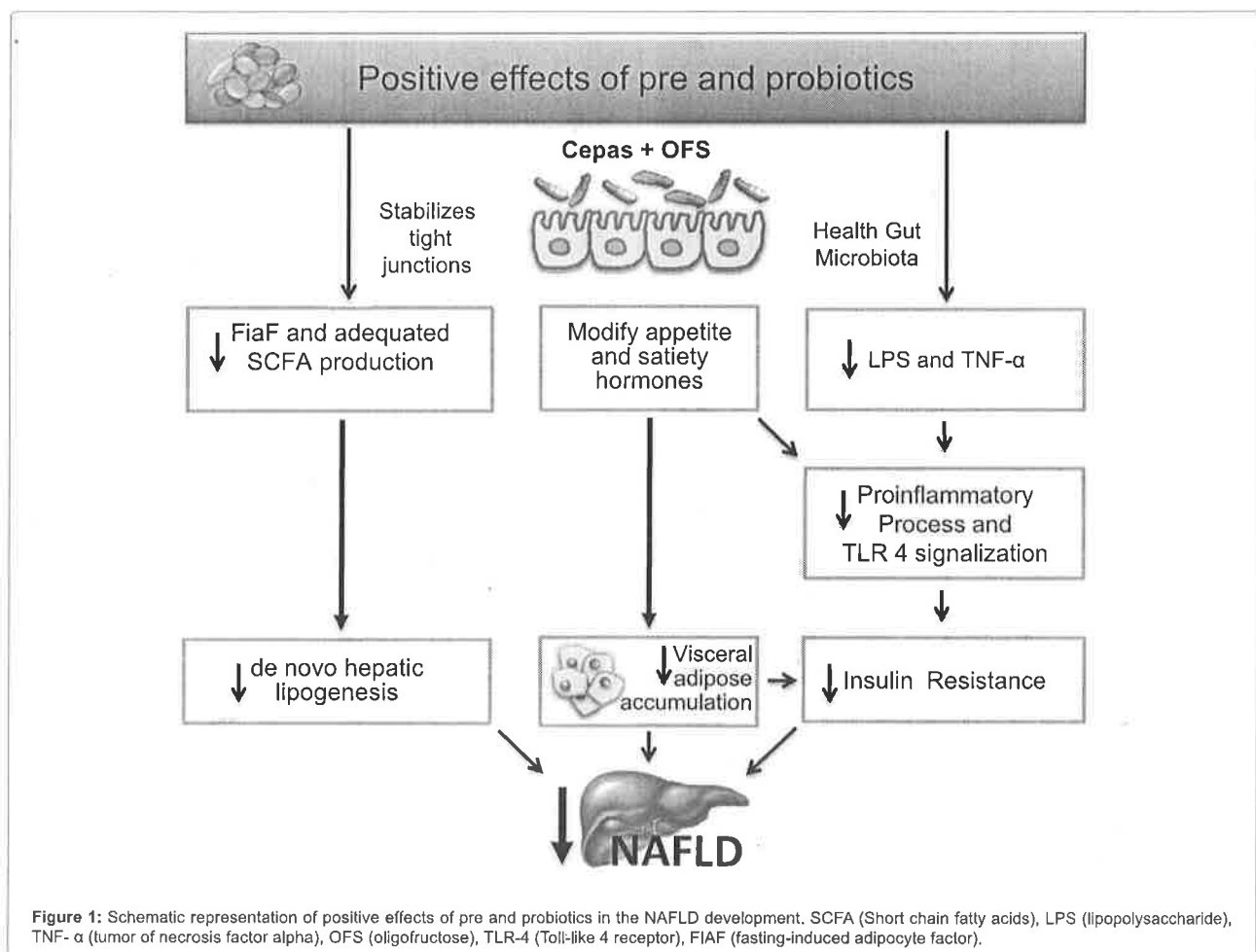
Dysbiosis in the gut microbiota refers to a condition with microbial imbalances in the bowel that may activate hepatic de novo lipogenesis by increasing the expression of lipogenic enzymes, acetyl co-A carboxylase (ACC), and fatty acid synthase (FAS) [64].

A recent study in obese rats showed that prebiotic fibers improve or normalize gut microbiota dysbiosis by decreasing Firmicutes and increasing Bacteroidetes phylae [82].

Finally, prebiotic induced changes in the gut microbiota also influence the production of the gut trophic hormone, glucagon-like peptide-2, which could potentially decrease the lipid and LPS concentrations due its effects on the intestinal permeability and epithelial tight junctions [85].

Prebiotic-rich diets may ameliorate NAFLD by attenuating de novo fatty acid synthesis as has been shown in animal models [86-89]. Fiordaliso et al. [89] verified that animals treated with oligofructose (OFS) have a modified liver capacity that can synthesize triacylglycerols from free fatty acids in isolated hepatocytes.

However, in another animal model of germ-free mice, there was a doubling of the hepatic triacylglycerol content and a concomitant



increase in the hepatic mRNA levels of sterol-responsive element-binding protein (SREBP-1) and carbohydrate-responsive element-binding protein (ChREBP), both of which are positive regulators of the aforementioned lipogenic enzymes [90,91].

Moreover, prebiotics associated with probiotics promote the production of short chain fatty acids (SCFA), leading to the growth of indigenous bifidobacteria and/or lactobacilli and lowering the luminal pH, which in turn prevents the growth of pathogens [92]. Acetate, propionate and butyrate are the main SCFAs produced in the large intestine. As described previously, although the greater part of butyrate is metabolized by colonocytes, propionate and acetate are delivered to the liver via the portal vein [93].

Butyrate is a major metabolite in the colonic lumen arising from bacterial fermentation of dietary fiber and is a critical mediator of the colonic inflammatory response. This SCFA is associated with the inhibition of HMG-CoA reductase, an enzyme involved in the metabolic pathway that produces cholesterol. Studies have shown that a diet enriched with prebiotic fibers promotes an increase in the ratio of propionate to acetate, decreasing lipogenesis as propionate inhibits lipogenesis, whereas acetate promotes the process [88,93].

Animals receiving a high fat diet and prebiotic treatment have decreased steatosis, fat storage in the white adipose tissue, systemic inflammation, and insulin resistance [93,94]. Prebiotic treatment in people results in a reduction in BMI, waist circumference, fat mass, and insulin resistance [81,95].

Prebiotic fiber supplementation is associated with reduced body weight or attenuated weight gain in lean, high-fructose fed high-fat, high-sucrose fed and genetically obese rodent models [96].

Although the majority of animal studies use a 10% inulin dose by weight with a minimum 4 week supplementation period, Sugatani et al. [97] reported reductions in the liver TAG and cholesterol in cafeteria diet-fed rats supplemented with 5% synthetic inulin for 3 weeks. In comparison, improvements in serum and liver lipids with prebiotic doses are reported to be as high as 20% [87]. The minimum inulin dose and necessary duration has not been fully defined and requires further study.

Human studies assessing the effects of prebiotic fiber supplementation on NAFLD patients are lacking; however, we can verify clinical protocols to identify the effects of prebiotic fiber supplementation on serum lipids. Parnell and Reimer [98] reported significant weight loss following 3 months of oligofructose supplementation in overweight and obese adults. Other possible mechanisms for the action of prebiotics in humans include improvements in glycemia and modifications to plasma glucagon-like peptide-1, peptide YY and ghrelin [82].

In summary, the prebiotic-induced, gut-mediated changes in luminal and peripheral metabolism include a reduction in bacteria-derived hepatotoxins, improved gut epithelial barrier, reduced inflammation, decreased de novo lipogenesis, modified appetite and satiety, and improved glycemic control [82] (Figure 1).

NAFLD and Vitamin D

Recent studies suggest that vitamin D is associated with obesity, diabetes, cardiovascular diseases, metabolic syndrome and NAFLD [99-101]. In a multiple logistic regression analysis study, performed on Korean men, the tertiles with lower 25(OH)D(3) levels presented with a significantly increased risk for NAFLD compared with the highest tertile, even after adjusting for the body mass index and metabolic syndrome. Accordingly, individuals with higher serum 25(OH)D3 presented with a significantly reduced risk for NAFLD independent of

obesity and metabolic syndrome [99]. Additionally, inadequate 25(OH) D status progressively increased the odds of NAFLD when classified categorically as sufficient (25(OH)D (>30 ng/mL), insufficient (15-30 ng/mL) or deficient (<15 ng/mL) [102].

In a recent meta-analysis, the vitamin D role in the pathogenesis of NAFLD was discussed. Deficiency of vitamin D, considering a cut-off serum level ranging from 12 to 30 ng/mL, was associated with a higher NAFLD risk. The normal range of vitamin D remains controversial; however, the most recent Institute of Medicine (IOM) Committee report endorses the use of 20 ng/mL [103].

The lower 25(OH) D concentrations in overweight and obese individuals needs to be confirmed and the possible reasons for differences in these concentrations must be studied in more detail to better manage the vitamin D status [104]. To prevent and treat vitamin D deficiency by maintaining serum 25(OH)D levels above 30 ng/mL, the Endocrine Society Guidelines recommend 400-1000 UI for children and 1000-2000 UI for adults [105].

The vitamin D is a hormone implicated in several aspects of metabolism and the human immune system [106]. This vitamin can be obtained from exogenous sources and can be synthesized in the skin by conversion of 7-two hydroxylation in the liver and kidney, generating active vitamin D (vitamin D, 1,25(OH)2D3) [107]. The hepatocytes are exclusively responsible for 25-hydroxylation, which is mediated by CYP27A1 and CYP2R1, two cytochromes expressed in the liver [108]. Barchetta et al. [109] demonstrated that the expression of these cytochromes in NASH patients is lower than in the control group, but this difference was not significant.

Moreover, liver vitamin D receptor (VDR) expression has a strong association with the NASH diagnosis independent of BMI, insulin resistance or adiponectin, suggesting a loss in the hydroxylation of hepatocytes and the hepatoprotective role of vitamin D [109]. The VDR is expressed in many tissues, such as the liver, pancreas, and several immune cells, and its expression is most abundant on the epithelial cells of the gastrointestinal tract. The VDR modulates the expression of several other genes that are involved in converting dehydrocholesterol to previtamin D3 by ultraviolet radiation [107]. The VDR is expressed by macrophages; 1,25(OH)2D3 upregulates the inhibitor of nuclear factor (NF)- κ B (I κ B- α) by increasing mRNA stability and decreasing I κ B- α phosphorylation, suggesting that 1,25(OH)2D3 has an anti-inflammatory effect on macrophages [110].

Vitamin D increases the VDR expression in the rat ileum and liver as well as in the ileum of humans [111]. The VDR stabilizes tight junctions in the intestinal epithelial cells [112], suggesting a possible reduction of LPS action and the proinflammatory process.

There is a negative correlation between VDR expressions on hepatocytes with the NAFLD activity score in humans [109]. In an animal study performed in rats fed on a high-fat/high-fructose corn syrup diet alone or with vitamin D depletion, the vitamin deficient group presented with significantly greater hepatic steatosis, lobular inflammation and higher hepatic messenger mRNA levels for TLR2, TLR4 and TLR9 and other proinflammatory and oxidative stress markers compared to the control group.

Vitamin D deficiency can exacerbate NAFLD through TLR activation accompanied by increased inflammation and oxidative stress [113]. In a recent investigation developed by our research group, vitamin D3 supplementation for a high fatty diet exerts an anti-inflammatory effect once it decreases the IL-6 production in epididymal adipose tissue in mice as well as in 3T3-L1 adipocytes stimulated with LPS [114].

Deficiency
Serum
CUTOFF

Another study developed in human adipocytes and in 3T3-L1 adipocytes reported decreases in the levels of inflammatory markers such as IL-6, MCP-1, and IL-1 β (mRNA and protein levels) after 1,25-dihydroxyvitamin D3 (1,25-(OH) $_2$ D $_3$) treatment. In basal and TNF- α -stimulated conditions, this treatment decreased the expression of the proinflammatory marker in 3T3-L1 and human adipocytes. Finally, the 1,25-(OH) $_2$ D $_3$ treatment promoted improvement in glucose uptake and AKT phosphorylation. These data support the involvement of the vitamin D receptor gene and NF- κ B in NAFLD, suggesting that low-grade inflammation could be linked to vitamin D deficiency [115].

The effects of 1,25(OH) $_2$ D $_3$ -VDR have not yet been fully defined, but it appears that vitamin D, likely cooperating with other regulators, exerts immunoregulation, antimicrobial defense, xenobiotic detoxification, anti-cancer actions, control of insulin secretion and, possibly, cardiovascular effects [110]. Indeed, the influence of vitamin D in the NAFLD mechanisms should be further explored [99].

The bioavailability of vitamin D is disturbed by obesity, wherein adiposity is associated with lower bioavailability of this vitamin, leading to impairment in insulin secretion and sensitivity [104]. Corroborating this finding, an investigation of two common VDR polymorphisms suggested that 2 major VDR gene polymorphisms may be linked to insulin secretion and resistance [107].

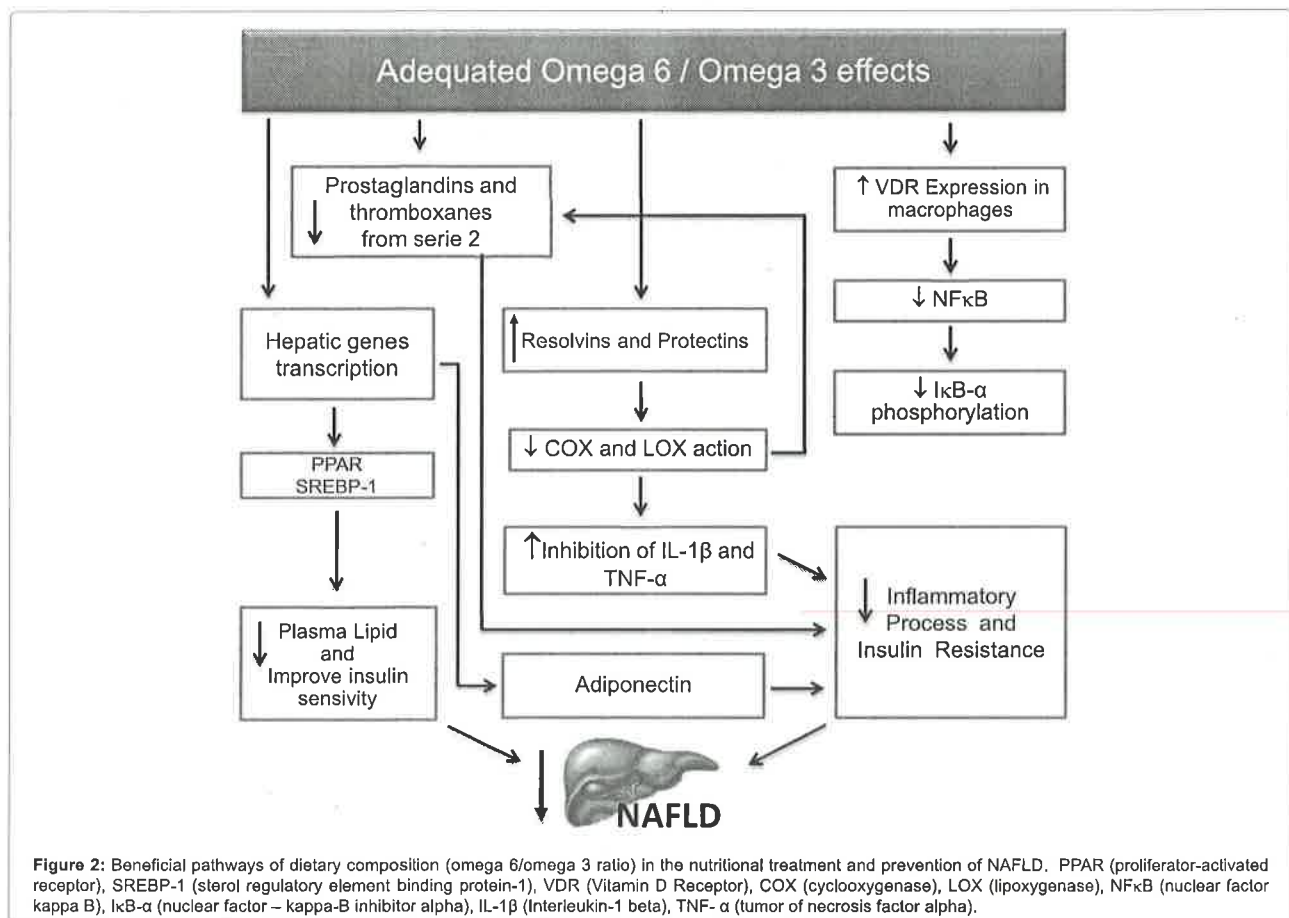
Pancreatic insulin secretion is inhibited by vitamin D deficiency,

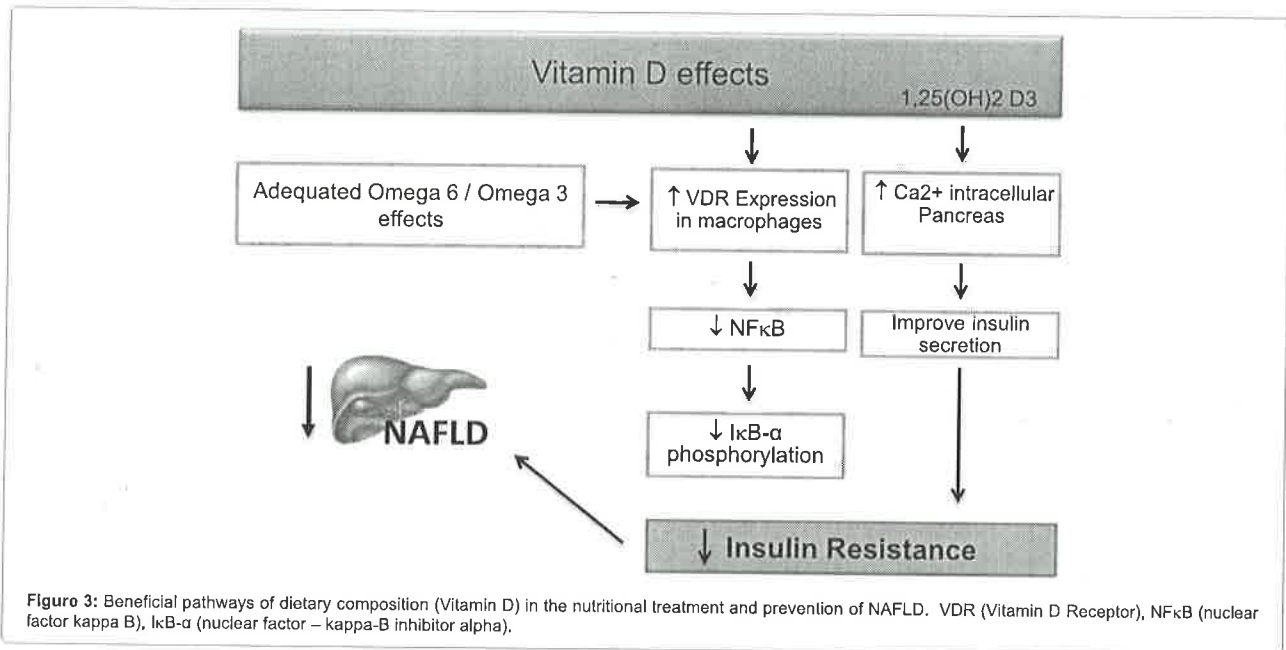
suggesting a role for this vitamin in the regulation of endocrine pancreatic function, especially in the β cell [116]. It is already known that 1,25(OH) $_2$ D $_3$ directly influences β cell insulin secretion through the induction of increases in the intracellular free calcium concentration through voltage-dependent Ca $^{2+}$ channels [117]. This mechanism is implicated in insulin cascade signaling, which exerts a fundamental role in preventing several diseases, such as NAFLD.

A study on young adults showed that the prevalence of metabolic syndrome components is significantly lower across quintiles of vitamin D intake, reinforcing the usefulness of vitamin D intake [100]. Vitamin D intake may modulate the risks of metabolic syndrome and, as cited previously, vitamin D is involved in the promotion of calcium influx, regulation of insulin secretion and glucose uptake [100].

Based on these findings, measuring vitamin D levels may be an important strategy in NAFLD treatment, and vitamin D supplementation may be associated with an adequate n3/n6 ratio and pre and probiotics, contributing to healthy gut microbiota [103] (Figures 2 and 3).

Finally, another possible mechanism linking vitamin D, the PUFAs and NAFLD has been studied. Researchers have identified several additional nutritional lipids as candidate, low-affinity VDR ligands that may function locally in high concentrations. The novel putative VDR ligands include w3- and w6-essential polyunsaturated fatty acids (PUFAs), docosahexaenoic acid (DHA) and arachidonic acid. The ligand binding of VDR triggers tight association between VDR and its





heterodimeric partner, retinoid X receptor (RXR), and only the VDR-RXR heterodimer can penetrate the deep groove of DNA and recognize vitamin D responsive elements (VDREs) in the DNA sequence of vitamin D-regulated genes. These VDR-RXR controlled genes encode proteins that determine bone growth and remodeling, intestinal calcium absorption, phosphate homeostasis, the mammalian hair cycle, cell proliferation, and lipid detoxification [110]. The n-3 PUFAs, such as DHA and EPA, and n-6 PUFAs, such as linoleic acid and arachidonic acid, compete with titrated 1,25(OH)₂D₃ for binding to VDR with affinities for the receptor that are four orders of magnitude lower than that of the 1,25(OH)₂D₃ hormonal ligand. Nevertheless, the authors concluded that high local concentrations of PUFAs could be present in select cells or tissues and, if VDR is expressed, result in VDR-mediated anti-proliferation/ pro-differentiation effects, which may partially explain the chemoprotective nature of diets rich in PUFAs [110].

New perspectives on the nutritional treatment and prevention of NAFLD

Knowledge of the inflammatory pathways involved in the development of NAFLD and new studies in nutrition, which reveal the strong positive influence of bioactive compounds in these inflammatory processes, are essential for guiding treatment and prevention recommendations. Based on these findings, we suggest the follow steps for NAFLD treatment:

Analyzing the dietary intake using different tools, such as the 24-hour recall, three-day food diary, food frequency and diet history, focusing on verifying the w3/w6 ratio, pre and probiotics and vitamin D ingestion;

The nutritional plan must be designed based on reducing the saturated and trans fatty acids and substituting monounsaturated and polyunsaturated fatty acids, corresponding to the following: saturated fat <7%, monounsaturated fat 10% and polyunsaturated fat 10% of the total energetic value. Supplementation with w3 (1 g/fish oil) is beneficial at a suggested ratio of n-6 to n-3 of approximately 3:1;

Considering that dietary composition can influence the health of

gut microbiota, the dietary plan should include daily alimentary sources of probiotics and a prebiotic-rich diet, such as with a 10% of inulin dose by weight with a minimum 4 week supplementation period. However, the optimal dose of pre and probiotics need to be further investigated.

Performing a serum analysis of vitamin D to verify possible deficiency and determine the necessary vitamin D supplementation;

Based on the cut-off serum level, 12 to 30 ng/mL of vitamin D was associated with an increased NAFLD risk, and the recommended vitamin D was 400-1000 UI for children and 1000-2000 UI for adults to correct the deficiency status and promote NAFLD improvement.

Altogether, these guidelines are important for nutritional therapy. Finally, the multidisciplinary team should always look for joint strategies and treat all aspects of the disease in question as well as delve deeper into promising areas such as nutrigenomics and translational investigations, which improve our understanding of the interaction between potential bioactive components and track the triggering of these comorbidities, all of which are new strategies for the treatment of NAFLD.

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